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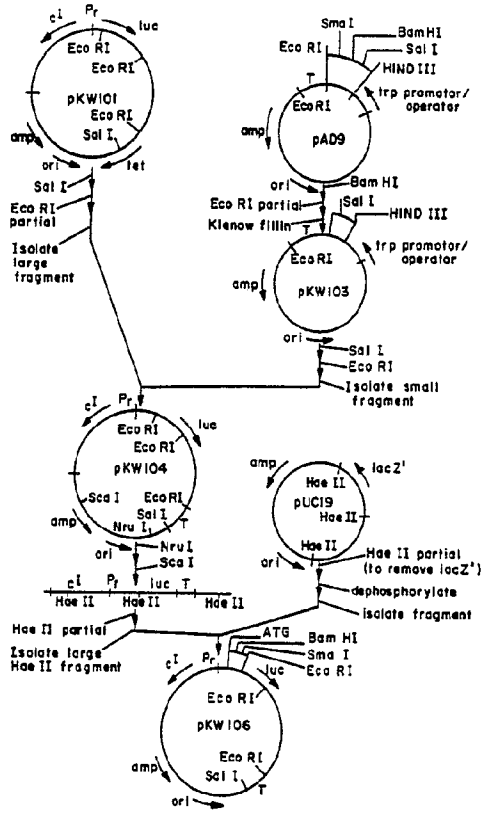
(54) Title: ISOLATION OF DNA SEQUENCES ENCODING LUCIFERASE ACTIVITY AND APPLICATIONS OF THE SAME

(57) Abstract

DNA compositions and methods of constructing and using the same consisting of DNA sequences encoding luciferase activity, or DNA sequences encoding hybrid molecules exhibiting luciferase activity and a second biological activity that are useful in performing biological assays.



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ISOLATION OF DNA SEQUENCES ENCODING LUCIFERASE ACTIVITY AND APPLICATIONS OF THE SAME

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BACKGROUND OF THE INVENTION

A large repertoire of assay methods are available for determining the presence of naturally occurring or synthetic molecules in biological fluids. Thus, it is commonplace in a commercial or academic laboratory for one to employ a battery of cytochemical, immunochemical, radiochemical, and physical chemical assays, or variations thereof, where detection of the substance of interest is premised on specific recognition by antibody, enzymatic reactions, viscosity changes, etc. Particularly popular are immunochemical assays reliant on antibody/antigen complex formation, and either radio or colorimetric detection of the complex.

Associated with each type of assay are advantages and disadvantages that are uniquely characteristic of that particular assay. For instance, radiochemical-based assays exhibit high sensitivity, yet present significant handling and storage problems. In contrast, the opposite is generally true for nonradiochemical-based assays; that is, they are less sensitive but do not have the handling and storage problems of radiochemical assays. Because of the

problems associated with radiochemical assays, considerable effort has been expended in order to develop assays as sensitive as radiochemical assays but without their drawbacks.

Bioluminescent assays theoretically offer the sensitivity of radiochemical assays but without the attendant problems; however, for several reasons they have not been widely utilized. Most such assays are based on light emission effected by the catalytic activity of luciferase with luciferin in the presence of ATP and molecular oxygen. The reaction consists of the conversion of luciferin to oxyluciferin with the concomitant generation of light. Thus, in lieu of using radio tracers, luciferase can be covalently attached to a ligand and then employed in any number of competitive binding assays. Nearly all bioluminescent assays employ luciferase isolated from either insects or bacteria, and luciferase from the bacterium Vibrio fischeri has recently been cloned. This bacterial luciferase consists of two different subunits. Because of its higher quantum efficiency and single subunit structure, insect luciferase is preferred over the multisubunit bacterial luciferase.

Unfortunately, there at least two problems associated with bioluminescent assays. First, luciferase is not readily available and easily isolated; and, second, the enzyme is easily denatured and hence rendered inactive by the chemical reactions necessary to effect covalent attachment to a ligand.

SUMMARY OF THE INVENTION

Methods for cloning and expressing regions of DNA that encode enzyme molecules with bioluminescence activity are described. The procedures make possible the isolation of a bioluminescent reporter molecule, luciferase, useful in virtually all biological assays, and additionally permits the molecular construction of dual-function hybrid molecules. The hybrid molecules uniformly exhibit luciferase activity, plus a second biological activity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the essential features of the plasmid, pKW101.

FIGURE 2 shows the production of plasmid, pKW104, from the plasmids pKW101, pKAD9, and pKW103, and the production of plasmid, pKW106, from pKW104 and pUC19.

FIGURE 3 shows the production of plasmid, pKW108, a cro-luc construct, from the plasmid pKJB824.11 and pKW106.

FIGURE 4 shows the production of plasmid, pKW109, from plasmids, pKWJB"Lac"ZI and pKW106.

FIGURE 5 shows a restriction map of the luciferase gene isolated from a genomic library.

FIGURE 6 shows a restriction map of the plasmid pJD200.

FIGURE 7 shows the plasmid construct, pJD201.

DETAILED DESCRIPTION OF THE INVENTION

The bioluminescence assay of the subject invention entails cloning and expressing a region of DNA that encodes a single protein subunit with luciferase activity, and then combining this region with other regions of DNA in suitable

expression systems to produce hybrid molecules useful in a variety of biological assays. Cloning of DNA sequences that encode luciferase activity can be accomplished by constructing either a cDNA or genomic library from an organism that exhibits such activity. Particularly useful for making a cDNA or genomic library is mRNA or DNA, respectively, isolated from the firefly Photinus pyralis, but it is to be anticipated that other insect species or worms that display the phenomena of bioluminescence can be suitably employed. Examples are Lampyridae, Elateridae, or Diptera.

Generation of a cDNA library is realized by isolating mRNA, particularly poly $(A)^+$ RNA by techniques well-known to those in the art, for example by chromotography on oligo[dT]-cellulose as described by Aviv and Leder in Proceedings of the National Academy of Sciences, U.S.A. (1972, Vol. 69, No. 6: 1408 - 1412), and reverse transcribing the poly $(A)^+$ RNA into cDNA. The cDNA can then be introduced into a suitable cloning vector and either transformed or transfected into procaryotic or eucaryotic cells. Any one of a number of vectors can be employed, including plasmids, cosmids, viral vectors, or hybrids thereof.

The general scheme for generating a cDNA library is to insert double-stranded cDNA made from mRNA into a suitable vector. This is conveniently accomplished by inserting the cDNA into a cloning site near a functional promoter and a translational initiation site on a suitable vector. This favored cloning site is present within the coding region of genes present on Escherichia coli plasmids or

bacteriophages. Several examples are well-known, particularly the β-lactamase gene of pBR322, the β-galactosidase (Lac Z) gene of pUC plasmids and λgtll, and the tryp genes of the tryptophan promoter plasmids. The insertion of cDNA results in a hybrid molecule, or fused polypeptides consisting of the NH2-terminal region of an E. coli protein covalently linked to a polypeptide and coded by the cloned cDNA fragment. If there are no "stop signals" separating the inserted cDNA fragment with the bacterial translation initiation signals, the hybrid RNA transcript is translated in the same "reading frame" as in the original mRNA. The fused molecule is then amenable to detection in cells that harbor the vector using a variety of immunochemical, or radiochemical nucleotide hybridization techniques.

Regardless of which vector is chosen to clone cDNA sequences encoding luciferase activity, it is necessary to select those cells that receive the vector from those that do not. In this regard, λgtll, a procaryotic expression vector described by Young and Davis in The Proceedings of the National Academy of Science (March 1983, 80:1194 - 1198), is attractive because it permits the construction and maintenance of large cDNA libraries, and ready detection of cells harboring cDNA regions with luciferase activity. In the λgtll system, double-stranded cDNA produced from mRNA isolated from bioluminescent insects is inserted into the restriction endonuclease Eco RI site in the E. coli Lac Z (β-galactosidase) gene carried by λgtll. This requires revealing ligation compatible nucleotide sequences on both the cDNA and gtll DNA to effect covalent bond formation

between the two. Generally, this can be realized by ligating Eco RI restriction endonuclease linkers to cDNA fragments and treating λgtll with Eco RI prior to inserting the cDNA into the phage. Lastly, λgtll containing foreign cDNA inserts is packaged *in vitro* to yield infectious phage particles and infected into a compatible strain of bacteria. After amplifying the cDNA library, λgtll phage that harbor DNA sequences encoding luciferase activity are identified by plating recombinant phage on a lawn of *E. coli* and inducing the production of hybrid proteins having luciferase and β-galactosidase sequences by adding an inducer of Lac Z transcription. The inducer enhances the level of hybrid protein production and therefore enhances the sensitivity of detecting cells harboring DNA sequences encoding luciferase antigenic activity. In many instances, addition of inducer will not be necessary as the level of hybrid protein production will be sufficiently great to be detected without its addition. A suitable inducer is isopropyldio-β-D galactopyranoside (IPTG).

Lac Z transcription directs the expression of foreign DNA inserts, that is proteins with luciferase properties. The latter are screened for luciferase antigenicity with antibody directed against luciferase by incubating the *E. coli* with antibody for a period of time sufficient to allow antibody binding to luciferase determinants. After a wash step to remove unbound antibody, bound luciferase antibody can be revealed by several techniques, one being to incubate the λgtll-infected antibody-treated lawn of *E. coli* with labeled-protein A of *Staphylococcus aureus*, or a second antibody directed against the luciferase antibody molecule.

The second antibody carries a suitable reporter molecule, such as radioactive atoms, or enzyme molecules.

A variety of radioactive and enzymatic reporter molecules, or complexes thereof, are available for use with the second antibody, examples being radioactive iodine, and the enzyme peroxidase, respectively. After allowing sufficient time for the second antibody to react with bound luciferase antibody, the lawn of cells is washed, and bound antibody revealed either by radioautography if radioactive atoms are used, or by the addition of a suitable chromogenic peroxidase substrate, particularly 4-chloro-1-naphthol if peroxidase is utilized. The latter is dissolved in a suitable solvent, methanol being effective, and then added to an aqueous solution containing H_2O_2. While the amount of the various substances can vary significantly without the detection of bound antibody being adversely affected, for optimal resolution about 60mg of 4-chloro-1-naphthol is dissolved in 20ml of methanol, which is added to 100ml of an aqueous solution containing 60µl of 30% H_2O_2. λgt11 recombinant plaques exhibiting luciferase antigenicity exhibit a purple color.

In addition to the aforementioned ways of detecting bound luciferase antibody, an alternate technique may be used that involves screening the cDNA library with a biotin-labeled second antibody, and subsequent formation of biotin-avidin peroxidase complex that is revealed by hydrolysis of a chromogenic peroxidase substrate. A suitable substrate is diaminobenzidine. This procedure is faster, requiring four hours to ascertain a positive plaque.

Luciferase antibody can be generated in rabbits as described by Green and McElroy in Biochemical Biophysical Acta (1956, 20:170 - 178) using purified firefly luciferase or by generating murine monoclonal antibodies against luciferase by the procedure of Kohler and Milstein as described in Nature (1975, 256:495 - 497), and Dosch and Gelfand in The Journal of Immunology (1977, 118:302 - 308). The latter references describes a procedure whereby murine monoclonal antibodies can be generated in vitro. Additionally, human monoclonal antibodies can also be produced by in vitro immunization techniques as described by Boss in Brain Research (1984, 291:193 - 195). In the case of rabbit serum containing luciferase antibodies, it is often necessary to separate from these antibodies other antibody populations that recognize E. coli or phage antigenic determinants, as well as luciferase antibodies that may cross-react with E. coli or phage. This can be accomplished by absorption of the undesirable substances to E. coli or phage using techniques described by de Wet et al. in DNA (1984, Vol 3, No. 6:437 - 447).

λgtll cDNA inserts were isolated preparative to identifying those that encode molecules with luciferase activity in a suitable expression vector, and to construct plasmids useful to express hybrid proteins. This was accomplished by isolating plaques that exhibit luciferase antigenicity, and the recombinant phage amplified by infecting a suitable strain of E. coli. DNA from recombinant λgtll phage was isolated from the desired plaque by one of several techniques, one being to isolate phage DNA from plate lysates performed as described in Maniatis,

Fritsch, and Sandbrook in Molecular Cloning (1982, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 371 - 372). Isolated recombinant phage DNA was then cut with Eco RI to release cDNA inserts. The latter were purified preparative to inserting the cDNA fragment into a suitable expression vector. Several methods can be used to purify the restriction fragment, particularly facile is the use of agarose gel electrophoresis onto a suitable paper substrate as described by Maniatis et al., supra.

To determine DNA inserts that encode protein with luciferase antigenic activity also exhibit luciferase enzymatic activity, cDNAs of various sizes were inserted into a host cell by transformation into procaryotes or transfection into eucaryotes, or into a suitable expression vector followed by transformation or by transfection. The latter procedure is more efficient and thus favored. A wide variety of procaryotic and eucaryotic vectors or "shuttle" vectors are routinely used for this purpose. The latter vectors are capable of replicating in both procaryotes and eucaryotes, as described by Struhl, Stinchcomb, Scherer, and Davis in The Proceedings of the National Academy of Sciences, U.S.A. (1979, 76:1035 - 1039). A general description of expression vectors and methods of using and isolating the same can be found by Grover in DNA Cloning, Volume II, A Practical Approach (1985, IRL Press, Oxford, Washington, D.C.) or by Maniatis et al., supra.

It is to be anticipated that cDNA inserts obtained from λgt11, or other vectors used to construct the initial gene library, that encode protein with luciferase antigenicity, will be of varying size. Moreover, it is to be further

anticipated that a number of different sized inserts may exhibit luciferase enzymatic activity. Regardless of the size of the insert, it can be assayed for luciferase activity and used to produce molecules with luciferase alone, or to construct hybrid molecules exhibiting luciferase activity, and antigenic and/or enzymatic activity. Regardless of cDNA size, cDNA expression is assayable by inserting the cDNA so as to come under the transcriptional and translational control mechanisms of either the vector employed and/or the host cell chosen to carry the vector. This procedure may require inserting suitable "linkers" into either the vector, the cDNA, or both. In addition to obtaining cDNA fragments that encode luciferase activity by excision from the vector initially used to produce the gene library, it may be desirable in certain instances to reduce the size of such cDNAs by restriction endonuclease or exonuclease treatment with one or more enzymes. As described above, these fragments can similarly be inserted into a suitable expression vector.

After inserting cDNAs exhibiting luciferase antigenic activity into suitable expression vectors, the expression product can be tested for luciferase enzymatic activity by preparing an extract of cells containing the cDNA insert, and adding to the extract the necessary co-factors to produce bioluminescence. Generally, this assay is performed in a buffered solution at near neutral pH containing magnesium chloride, luciferin, and adenosine triphosphate. Light emission was detected with a luminometer.

In addition to ascertaining DNA sequences that encode luciferase activity by generating a cDNA library, it is

possible to discern such sequences by constructing a genomic DNA library. This is accomplished by isolating DNA from a suitable bioluminescent insect or worm, particularly P. pyralis, and then fragmenting the DNA into fragments that are up to 20 kilobases long. This can be accomplished by using one or more restriction endonucleases followed by ligating the fragments into a suitable vector at a restriction site as described for generating a cDNA library. A useful restriction endonuclease is Sau3A, which yields 12 - 20 kilobase DNA fragments when the DNA is partially digested.

While digestion with DNA restriction endonucleases allows for ready cloning of the fragments, and thus is the preferred method of fragmenting insect DNA, it is possible to obtain fragments by subjecting DNA to destructing forces, particularly sonication or mechanical agitation. The size of the DNA fragments dictates the ease and convenience of cloning; and while there is anticipated to be a lower upper limit comprising a DNA sequence that encodes luciferase activity, it appears that a fragment in the range of about 20 kilobases or less will dictate the type of cloning system employed.

Cloning of genomic DNA fragments to produce a genomic DNA library can be effected in a variety of plasmids, cosmids, viral vectors, or hybrids thereof, similar to those used to produce a cDNA library. Vectors capable of replicating in either procaryotes, eucaryotes, or both, that is shuttle vectors, are similarly utilized to generate a genomic library. Shuttle vectors are the preferred recipients of the DNA fragments as they permit growing the

vector in usable quantities in bacteria, and then testing for the presence of the desired DNA sequence in eucaryotes. Regardless of the vectors used, all should contain one or more selectable markers. After DNA fragments are inserted into a suitable vector and the vector inserted into a host cell and the latter selected onto identified cells containing inserts, the genomic library is amplified and cells harboring DNA luciferase sequences identified. This can be achieved using immunochemical antibody detection techniques if the sequences are inserted into vectors compatible with such screening, such as pUC plasmids, λgtll, or the like. Alternatively, DNA luciferase sequences can be ascertained using tracer-labeled homologous nucleotide probes. The latter can be generated by a variety of techniques well-known to those skilled in the art, particularly ^{32}P nick-translation techniques of cDNA-cloned probes with luciferase sequences, or by synthesizing an oligonucleotide probe homologous to luciferase DNA sequences. The latter probes are derived from a knowledge of luciferase immunoacid sequences translatable into nucleotide sequences via the genetic code. In addition other less facile techniques are available for defining genomic DNA sequences such as employing labeled luciferase mRNA. Once cells are identified that harbor a recombinant vector exhibiting DNA luciferase sequences, the DNA from these structures can be isolated by techniques well-known in the art.

It should be noted that while cloning of either cDNA or genomic DNA sequences with luciferase activity can be readily achieved in E. coli-based plasmids or their phage,

that a variety of other vectors are usable, and may even be preferred. For example, Mizamoto, Smith, Farrell-Towt, Chizzonite, and Summers in Molecular and Cell Biology (1985, 5:2860 - 2865) have shown that a baculovirus vector produces high levels of foreign protein in an insect cell line. Thus, since one possible luciferase that can be cloned by the methods of the subject invention is obtainable from insects, DNA sequences encoding the latter may be more stably in this system.

Regardless of whether a cDNA or genomic library is used to identify DNA sequences that encode luciferase activity, DNA inserts can be isolated from either library and used by themselves or in combination to construct expression vectors that produce hybrid molecules, particularly proteins that exhibit both luciferase activity and a second functionality. This can be accomplished by isolating the cDNA or the genomic DNA inserts and ligating either into a suitable expression vector at a site near a functional promoter and a translational initiation site on the promoter. The latter, of course, exhibits cell-selectable markers, as well as the necessary replication and regulation features associated with growing such vectors and expressing DNA inserts contained therein. Examples of such are genes that code for drug resistance, enhancers upstream activation sites (UASs), as well as transcriptional control units that are not found on the upstream, i.e., the 5', side of the genes regulated.

Additionally, once a vector has been obtained that contains either a cDNA or genomic DNA fragment or hybrid thereof that encodes or expresses luciferase activity, the DNA sequence can be mutated using a variety of techniques

and chemicals to yield chemicals with altered luciferase activity. This can be accomplished by techniques well-known in the art, particularly those described by Smith in The Annual Review of the Genetics (1985, pp. 423 - 462). Thus, DNA can be deleted from either the 5' or the 3' ends of the molecule, or mutagens such as sodium bisulfite can be employed. Following selection of cells harboring the mutant molecules, the latter can be isolated and assayed for features not associated with native luciferase. Such might be altered specificity for ribonucleoside triphosphates. Native luciferase utilizes adenosine triphosphate, and it can be expected that the molecule exhibiting guanosine triphosphate hydrolysis specificity will be obtained. Additionally, mutant molecules that exhibit altered light emission properties can also be expected to be produced by these techniques.

In addition to inserting cDNA, genomic DNA, or hybrids constructed thereof encoding luciferase activity into bacteria or eucaryotic cells by expression vectors, the same can also be inserted into plants by one of two routes. First, a variety of vectors primarily based on Ti plasmids isolated from the bacteria Agrobacterium tumefaciens can be utilized. This involves inserting DNA sequences encoding luciferase activity downstream of a suitable plant cell promoter, transforming the bacterium with the recombinant plasmid, and then infecting plant cells with the bacterium. The most commonly used plasmids are the octopine or nopaline types. This procedure is preferred for dicotyleydonous species. The types of vectors available, including shuttle vectors, as well as their properties are described in An,

Watson, Stachel, Gordon, and Nester in The Journal of the European Molecular Biological Organization (1985, 4:277 - 286) and Caplan, Herrera-Estrella, Inze, Van Haute, Van Montague, Schell, and Zambryski in Science (1984, 18:815 - 821) and Fraley et al. in Biotechnology (1985, 3:629 - 635) and Klee, Yanofsky, and Nester in Biotechnology (1985, 3:637 - 642).

A second method for transferring DNA sequences encoding luciferase is to electroporate vectors containing the same directly into plant cells. This procedure complements Agrobacterium invection as it permits the transformation of monocotyledonous plant species.

The production of recombinant vectors that express molecules with luciferase activity and/or hybrid molecules that exhibit luciferase activity and a second biological activity are useful in a variety of biological assays. For example, luciferase can be employed to measure the amount of biomass present in a self-propagating system as described by De Luca and McElroy in Methods in Enzymology (1978, Academic Press, New York, 57:3 - 15).

Hybrid molecules exhibiting luciferase activity and a second protein capable of binding to cell associated cytoplasmic or plasma membrane structures can be expected to be favorably employed in a variety of cytochemical assays. For instance, it is possible to construct an expression vector exhibiting sequences that encode the antibody binding site of Protein A as the latter are described by Colbert, Anilionis, Gelep, Farley, and Breyer in Journal Biological Response Modifiers (1984, 3:235 - 259), and using the same in combination with DNA sequences encoding luciferase activity to produce a hybrid molecule with bioluminescence

and antibody binding activity. Thus, using routine biochemical materials and methods, it is possible to use hybrid molecules exhibiting luciferase activity and Protein A binding activity to detect cell surface or cytoplasmic antibody via the Protein A antibody binding portion of the molecule, and detecting the amount bound, or the cell types that exhibit binding, by assaying for bioluminescence emitted by the luciferase active portion of the molecule in the presence of its requisite substrates.

Construction of hybrid molecules exhibiting luciferase activity and a second biological activity permit the use of the same in a wide variety of immunochemical, cytochemical, etc., assays. For instance, antibodies can be raised against the nonluciferase region of the molecule and used in standard solid or liquid phase immunoassays to detect the presence of molecules with similar antigenicity in biological fluids. In this instance, detection and quantitation of the latter would be based on the intensity of light emission from the luciferase portion of the hybrid molecules.

EXAMPLES

Example 1. Construction of a cDNA Library from P. pyralis.

Preparatory to creating a cDNA library, total RNA was isolated from the lanterns of fireflies, P. pyralis. The tissue was homogenized in guanidinium thiocyanate, and the RNA pelleted through a cesium chloride cushion as described by Chirgwin, Przbyal, MacDonald, and Rutter in Biochemistry (1979, 18:5294 - 5298). Poly $(A)^+$ RNA was isolated by

chromotography on oligo(dT)-cellulose as described by Aviv and Leder in Proceedings of the National Academy of Science, U.S.A. (1972, 69:1408 - 1412). Next the poly(A)$^+$ RNA was eluted from the column with water and then precipitated with 1/10 volume of 3 molar sodium acetate and 2.5 volumes of ethanol were added. The RNA was precipitated overnight at $^-$20°C and pelleted. The pellet was washed with 70% ethanol, lyophilized, and the poly (A)$^+$ RNA was dissolved in water and frozen.

The poly (A)$^+$ RNA was used to generate cDNA as described by Maniatis, Fritsch, and Sandbrook in Molecular Cloning (1982, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 229 - 246) with the following modifications. Unlabeled deoxyribonucleoside triphosphates were present during first-strand synthesis at 200μM, poly (A)$^+$ RNA was used at 50μg/ml, and reverse transcriptase at 2000 units/ml (obtained from Boehringer Mannheim Biochemicals), and [α-^{32}P]dCTP at 250μcuries/ml. Additionally, second-strand cDNA was synthesized by the technique of Gubler and Hoffman in Gene (1983, 25:263 - 269). This procedure employs RNase H and DNA polymerase 1, and has the advantage of generating cDNA libraries from submicrogram quantities of poly (A)$^+$ RNA as it eliminates the vector primer system and the classical hairpin loop S1 nuclease cleavage steps, which result in low cloning efficiency.

Nucleotides were removed after the synthesis of the first and second strands of the cDNA by ethanol precipitation and a wash with 70% ethanol. Next, double-stranded cDNA was treated with Eco RI methylase, the latter obtained from New England Biolabs. Phosphorylated Eco RI

linkers (pGGAATTCC) also obtained from New England Biolabs were ligated to the double-stranded cDNA in 66μM Tris pH 7.5, 6.6μM magnesium chloride, 10μM dithiothreitol, and 1μM ATP. λgt11 DNA was prepared by first ligating the cohesive ends and cutting at the unique Eco RI site. The Eco RI ends were dephosphorylated to prevent spontaneous rejoining with alkaline phosphatase using the conditions described by Maniatis, Fritsch, and Sandbrook in Molecular Cloning (1982, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 133 - 134).

Next, 0.5μg of linker cDNA was ligated to 10μg of dephosphorylated, Eco RI-cut λgt11 with 7 Weiss units of T4 DNA ligase, also obtained from New England Biolabs. The reaction was carried out for 12 hours at 4°C in a total volume of 10μl. The ligated λgt11 phage was packaged in vitro according to the protocol of the supplier of the λ-packaging kit, which was obtained from Bethesda Research Laboratories. The cDNA phage library was titered on E. coli strain Y1088 as described by Young and Davis in Science (1983, 22:778 - 782) on plates containing the chromogenic substance 5-bromo 4-chlor 3-indolyl β-D-galactopyranoside and 1mM isopropyl thio-β-D-galactopyranoside (IPTG). Since insertion of DNA sequences into the Eco RI site of λgt11 inactivates the E. coli Lac Z (β-galactosidase) gene, the proportion of phage carrying cDNA inserts can be determined by plating dilutions of Y1088 in the presence of IPTG and X-gal. Under these conditions, λgt11 without inserts produces blue plaques, whereas phage carrying cDNA produces white plaques. Using this method, it was determined that packaging of lug of λgt11 DNA ligated to cDNA yields approximately 100,000

recombinant phage, and after amplification of the library on Y1088, the recombinants represented approximately 10% of the total phage population.

Example 2. Identification of cDNA Sequences That Encode Molecules with Luciferase Antigenic Determinants.

The λgt11 phage library described above was plated on a lawn of E. coli strain Y1090 [Δlac V169 proA$^+$ Δ lon araD139 strA supF trpC22:Tn10(pMC9)], and induced with isopropyl thiol-β-D-galactopyranoside as described by Young and Davis in Science, supra. The plate containing the lawn of phage-infected bacteria was overlaid with an inducer-impregnated nitrocellulose filter. Plates containing overlaid filters were incubated at 37°C for 2 to 8 hours. The position of the filter was marked with a needle so that it could later be realigned with plaques on the dish. The filter was then transferred to a clean petri dish and washed with buffer containing 0.17M NaCl, 0.01M Tris pH 7.5 (TBS) for 5 minutes. The phage plate from which the filter was taken was stored inverted at 4°C until needed.

The TBS buffer was removed from the filter and 5ml of the same buffer plus 3% gelatin with a final pH of 7.5 was added to the filter, and agitated for 15 minutes and drained. Enough TBS buffer containing 3% gelatin, 0.02% azide, and 2μg/ml of IgG specific luciferase antibody was added to each filter, which were then gently shaken overnight to permit the antibody to absorb to luciferase antigenic determinants. Next, the filters were washed for 10 minutes with 10ml of TBS buffer, then 5 minutes with 10ml of the same buffer plus 0.05% NP-40, a nonionic detergent,

followed by another 10ml of TBS wash. To each plate containing filters was added 7ml of TBS plus 3% gelatin, and 5µl of peroxidase conjugated goat-antirabbit IgG obtained from Bio Rad Corporation. Plates were gently agitated for 1.5 hours to permit sufficient absorption of the peroxidase conjugate to bind to the first antibody and then washed as described above for absorption of the first antibody.

Plaques that adhered to the filters and that exhibit luciferase antigenic determinants were visualized by developing the filters using the chromogenic peroxidase substrate, 4-chloro-1-naphthol. 60ml of the latter were dissolved in 20ml of ice-cold methanol, the latter being mixed just before use with 100ml of TBS containing 60µl of 30% H_2O_2. To each filter was added enough of the developing solution to cover the filter; and generally within 10 to 15 minutes if a particular plaque was positive for luciferase antigenic binding, it exhibited a purple color. Those plaques that exhibited positive staining were located on the phage plates, and removed from the plate using a sterile Pasteur pipette. Phage were eluted from the agar in which they were embedded by transferring them to a solution containing 0.1 molar sodium chloride, 50mM Tris, ph 7.5, 10mM magnesium sulfate, 0.01% gelatin, and 2 drops of chloroform, and were allowed to elute overnight at 4°C.

Using the above-described procedures, it is possible to isolate λgt11 recombinant phage containing cDNA inserts that encode for protein containing luciferase antigenic determinants.

Example 3. Preparation of Anti-luciferase Antibody.

Firefly luciferase was purified from adult lanterns of P. pyralis according to the procedure of Green and McElroy in Biochemistry Biophysical Acta, supra. The enzyme was crystallized 4 times and used to immunize New Zealand White Rabbits. The antiserum was fractionated using classical ammonium sulfate precipitation techniques at a concentration of 0% - 25% and 35% - 37%. The resulting pellet obtained from using 35% - 37% ammonium sulfate was dissolved in a minimal amount of water and dialyzed against the same. The solution was centrifuged and the pellet discarded. Next, the solution was dialyzed against 0.01 molar of sodium phosphate or Tris-Cl buffer, 0.15 molar sodium chloride, pH 7.8 containing 0.02% sodium azide to inhibit bacterial growth.

Example 4. Expression of cDNA Sequences Encoding Molecules with Luciferase Activity in Procaryotes.

λgt11 DNA obtained from plate lysates was partially digested with the restriction endonuclease Eco RI, and the cDNA inserts released were isolated by agarose gel electrophoresis yielding inserts ranging in size from 400 base pairs to 1800 base pairs. Isolation of recombinant phage DNA was conducted as described by Maniatis et al., supra. The 1.8 kilobase fragment was ligated into a procaryotic expression plasmid, pKJB824.17. The properties of this plasmid, as well as the conditions needed to propagate it, are described by Buckley in Ph.D. Thesis (1985, University of California, San Diego). pKJB824.17 exhibits the bacteriophage temperature-sensitive repressor cI_{857}, and the Rightward promoter, P_r followed by a

truncated cro gene exhibiting an Eco RI site. Thus, the homology of the nucleotide bases of the 1.8 kilobase fragment produced by Eco RI cleavage of the recombinant phage, permits ligation of the fragment into the Eco RI site of pKJB824.17 resulting in fusion of the reading frames of the truncated cro gene to the 1.8 kilobase cDNA fragment. The resulting novel plasmid is termed pKW101 and is shown in Figure 1.

pKW101 was transformed into the E. coli strain TB1[ara,Δ(lac,proAB),strA,Ø80lacZΔM15,hsr^-,hsm^+] using the calcium precipitation technique described by Maniatis et al., supra. TBI cells containing pKW101 cells were selected by their ability to grow in drug-supplemented media, and then grown up in 10ml of LB broth containing 10g of tryptone, 5g yeast extract, and 5g sodium chloride per liter with pH 7.4. Cells were grown at 30°C to an optical density of 0.8.

Next, the expression of luciferase activity was achieved by inactivating the λ repressor by heating the cells to 45°C for 30 minutes followed by further incubation for 1 hour at 37°C. Cells were collected by low-speed centrifugation and resuspended in 200µl of 100mM KPO_4 pH 8.0, 2mM EDTA, 1mM dithiothreitol, 0.2mg/ml protamine sulfate, and 1mg/ml lysozyme on ice for 10 minutes. They were frozen on dry ice and thawed to effect cell lysis and hence liberate molecules with luciferase activity. Aliquot of the cell extract were assayed for luciferase activity by adding 50µl of the extract to 300µl of 25mM glycylglycine buffer pH 7.8, containing 5mM magnesium chloride and 0.1mM luciferin. Bioluminescence was ascertained by placing the

tubes in luminometer equipped with a chart recorder, and then 100µl of 20mM ATP, pH 7, was injected. The intensity of light emission was recorded, a flash of yellow-green light being observed that rapidly decayed to a lower level of luminescence.

To insure that the luminescence observed was due to the expression of inserted cDNA, several controls were run. Luciferin alone added to the cell extract yielded a low level of light, presumably because of endogenous levels of ATP in the cells. In contrast, luciferase activity is not detected in extracts of heat-induced TB1 cells or TB1 cells carrying the vector pKJB824.17 absent cDNA inserts.

Example 5. Construction of Plasmids Useful for Generating Proteins Exhibiting Dual Functions.

Generation of hybrid proteins that exhibit luciferase activity and a second functionality involve engineering a plasmid that permits cloning of functionally distinct DNA sequences adjacent each other that are read in phase. A suitable plasmid, pKW106, can be constructed by treating the plasmid pKW101 with the restriction endonucleases Sal I and Eco RI so as to remove DNA sequences that encode tetracycline resistance in anticipation of inserting therein a transcription terminator sequence derived from another plasmid. Thus, pKW101 minus the region of DNA containing tetracycline-resistance was isolated by agarose electrophoresis. This reaction scheme is shown in Figure 2. Next, to derive the transcription terminator sequences, the plasmid, pAD9 (also shown in Figure 2), was treated with the restriction endonucleases Bam HI, Eco RI (partial

digestion), and then the recessed 3' prime ends created by cleavage with Eco RI and Bam HI filled in with the Klenow fragment of E. coli DNA polymerase 1 as described by Maniatis et al., supra. The resulting plasmid, termed pKW103, was further treated with Sal I and Eco RI, and the small restriction fragment exhibiting the transcription terminator isolated by agarose gel electrophoresis.

The large fragment generated from Sal I and Eco RI (partial) digestion of pKW101, and the small fragment generated from pKW103 containing the transcription terminator were ligated to produce the plasmid, pKW104.

Finally, plasmid, pKW106, was generated by treating pKW104 with the restriction endonucleases Nru I and Sca I causing the release of a fragment containing cI, P_r, the cDNA insert encoding luciferase activity, the transcription termination sequences, and 3' and 5' regions of plasmid DNA. This fragment exhibits the three Hae II sites--one near cI, a second in the DNA region encoding luciferase activity, and the third downstream of the transcription terminator sequence. This fragment was partially digested with Hae II and inserted into the plasmid pUC19, which previously was prepared by partial digestion with Hae II cutting to remove the Lac Z gene contained therein. The result is the plasmid, pKW106, shown in Figure 2.

Several features of pKW106 are worth noting. First, it contains in sequential order restriction endonuclease sites for Bam HI, Sma I, and Eco RI immediately downstream of the phage promoter, P_r. The DNA region encoding luciferase activity is under control of the phage promoter, P_r, and is adjacent the transcription terminator region. Further, the

plasmid carries the gene for ampicillin resistance. The restriction sites Bam HI and Sma I are particularly useful in that they are unique and provide sites for inserting cDNA to produce hybrid protein molecules that exhibit luciferase activity plus a second biological activity. Eco RI can also be used for this, but is not unique and requires partial digestion.

Example 6. Construction of Plasmids Exhibiting DNA Sequences Encoding Hybrid Proteins.

Representative members of two types of plasmids that have been constructed to date that express hybrid protein molecules will be described.

a. Hybrid Proteins Exhibiting Modified Luciferase Bioluminescence.

The plasmid pKW106 was produced as described in Example 5 and treated with Eco RI to release the DNA insert containing the sequence encoding luciferase activity. The sequence was isolated using standard techniques and ligated to the plasmid, pKJB824.11, produced as described by Buckley, supra after the latter was cut with Eco RI and dephosphorylated with calf intestine alkaline and phosphatase to prevent recirculization and subsequent ligation without a DNA insert. The result is plasmid, pKW108, which is shown in Figure 3.

When pKW108 is transformed into a suitable strain of E. coli, it expresses a hybrid protein consisting of 20 amino acids of the cro gene, with the remainder being derived from the DNA sequences encoding luciferase activity.

When extracts of cells containing pKW108 are assayed for bioluminescence as described in Example 4, an initial flash of light is observed, which decays rapidly to 40% of the maximum intensity. Surprisingly, there then follows a period wherein the intensity of light emitted remains constant until the luciferin substrate is depleted. This long-lived period of light emission should be compared with the near instantaneous and complete decay of bioluminescence effected by native luciferase.

b. Hybrid Protein Molecules That Exhibit Luciferase and a Second Enzymatic Activity.

Representative of a class of plasmids that express hybrid molecules that exhibit two distinct biological activities is pKW109. This plasmid contains DNA that encodes a hybrid protein exhibiting luciferase activity. Figure 4 shows the steps taken to generate pKW109. The plasmid pKW106 described in Example 5 was restricted with Bam HI and Sca I, and the large fragment containing the DNA sequences, including luciferase activity and the transcription terminator, were isolated. Next, the plasmid pKJB"lac"ZI was cut with Bam HI and Sca I. Restriction with Bam HI and Sca I liberates a fragment with cI, P_r and the Lac Z-mc5 DNA sequences. The latter was ligated to the Bam HI/Sca I DNA fragment produced from pKW106 to yield pKW109.

Expression of pKW109 in a suitable strain of E. coli can be expected to yield a protein with one enzymatic activity and luciferase, both of which are assayed by techniques described herein, or well-known in the art.

pKW109 demonstrates that a large peptide can be fused to Luc without destroying bioluminescent activity. Thus fusions with two enzymatic activities are feasible.

Example 7. Immunoassay Employing Hybrid Luciferase Molecules.

Representative of the types of assays that hybrid molecules that exhibit luciferase can be employed in are solid-phase immunochemical binding assays to detect antigenic substances in biological fluids. Thus, it should be possible to determine the presence of β-galactosidase using the hybrid molecule, exhibiting luciferase enzymatic activity and β-galactosidase antigenic activities generated in Example 6. This can be accomplished by raising antibodies to β-galactosidase; either polyclonal or monoclonal antibodies can be utilized. Polyclonal antibodies can be generated in rabbits and purified by standard ammonium sulfate precipitation procedures, whereas mouse monoclonal antibodies can be prepared by the procedure of Kohler and Milstein as described in Nature (1975, 256:495 - 497). Antibodies so obtained are affixed to solid support surfaces using techniques well-known in the art, cellulose or agarose beads being suitable for this purpose. Preparation of the beads by cyanogen bromide activation and subsequent β-galactosidase antibody coupling is carried out as described Wide in Methods of Enzymology (1981, 73:203 - 224). Next, beads containing coupled antibody are added to test tubes with a suitable buffer, and a cell extract or other source of fluid containing an unknown amount of β-galactosidase, and the luciferase β-galactosidase hybrid

molecule added. The reactants are allowed to compete for binding to antibody bound to the bead for 1 hour at 37°C, then the beads separated from unbound reactants by centrifugation. After washing the beads with a suitable buffer to remove residual amounts of unbound reactants, the amount of β-galactosidase present in the cell extract is determined by assaying the intensity of bioluminescence generated from the luciferase moiety bound indirectly to the beads by attachment of the β-galactosidase region to the antibody. This is accomplished by performing a luciferase assay as described in Example 1 and monitoring light intensity. Light intensity is related to β-galactosidase concentration in the cell extract by simultaneously constructing a standard curve by repeating essentially the same assay but using a known amount of β-galactosidase, and determining the concentrations of β-galactosidase needed to successfully compete with the hybrid molecule to yield a particular level of light emission. The procedures, buffers, and reaction conditions necessary to carry out these assays is further described by Wide in Methods of Enzymology, supra.

Example 8. Construction of a Genomic Library from P. pyralis.

DNA from P. pyralis was isolated from frozen beetles by grinding the same in a mortar and pestle. 50 mM Tris, pH 8, and 10 mM EDTA were added and proteinaceous material digested with proteinase K in the presence of 1% sodium dodecyl sulfate (SDS). DNA was twice extracted, once with phenol-chloroform and a second time with chloroform, and

precipitated with ethanol and redissolved in TBS buffer. The DNA was subsequently purified by banding on an ethidium bromide-cesium chloride equilibrium gradient. After isolating the same from the gradient, DNA was subjected to partial digestion with the restriction endonuclease Sau3A. Fragments ranging in size from 12 - 20 kilobases were isolated by centrifugation on a sucrose-density gradient. These fragments were ligated to the λ vector EMBL4 DNA, which was previously digested with Bam HI. This phage mixture was packaged using λ *in vitro* packaging extracts, which are commercially available form Bethesda Research Laboratories, or Vector Cloning Systems. Packaged phage were then plated on a lawn of *E. coli* cells, strained LE392, and plaques so obtained were screened by the filter hybridization method of Benton and Davis as described in *Science* (1977, 191:180 - 182). The probe used to detect plaques harboring luciferase DNA sequences was a 5' Eco RI fragment obtained from the cDNA library discussed in Example 4. This probe, termed Luc23, was labeled with ^{32}P by nick-translation as described by Rigby, Diekman, Rose, and Berg in *Journal of Molecular Biology* (1977, 113: 237 - 251). Plaques were detected that hybridized to the Luc23 cDNA probe, and these were purified by standard techniques. All the clones obtained contained three Eco RI fragments that were homologous to the luciferase Luc23 cDNA probe. A restriction map of the luciferase gene is shown in Figure 5.

Example 9. Construction of Vectors Suitable for Expressing Luciferase Activity in Eucaryotic Cells.

To obtain expression of luciferase activity in eucaryotic cells, a hybrid DNA molecule was constructed consisting of Luc23 cDNA and genomic DNA. This was accomplished by purifying the 5' Eco RI fragment from the genomic clone obtained as described in Example 8. The procedure entailed using Eco RI to obtain the fragment, thus it was necessary to fill in the Eco RI sites to create blunt ends prior to ligating Hind III linkers to the termini of the fragment. Next, the Hind III-linkered fragment was inserted into a Hind-III site of the plasmid vector pUC13 to yield the plasmid pJD200. A restriction map of pJD200 is shown in Figure 6.

Digestion of pJD200 with XbAI releases all but the 5'-most sequences of the luciferase genomic DNA from the vector. Thus, when pJD200 was digested with Bam HI and XbAI, it was possible to isolate the fragment consisting of the vector plus the 5'-most portion of the luciferase gene. This fragment was ligated to a Bam HI-XbAI fragment composed of luciferase cDNA extending from the same XbaI site that was present in the genomic DNA to the end 3' of the Luc23 cDNA to yield the plasmid pJD201. Unlike the Luc23 cDNA, which lacks an initiation codon, pJD201 has the natural luciferase translational start and polyadenylation site. Plasmid pJD201 is shown in Figure 7. It should be noted that the Luc23 cDNA/genomic insert of pJD201 is suitable in use with a wide variety of vectors that may be expressed in eucaryotes and procaryotes. Further, it should be noted

that the hybrid DNA molecule can also be utilized for expressing luciferase activity in plant cells.

Example 10. Example of Luciferase Activity in Eucaryotic Cells.

The Hind III-Bam HI DNA fragment exhibiting luciferase sequences was obtained from pJD201 described in Example 9 using the same enzymes, and was inserted downstream from an SV 40 promoter in the plasmid pBR322-based vector pSV2. The new construct, termed plasmid $pSV2_L$ was introduced into CV 1 monkey cells by the calcium phosphate DNA precipitation technique. After 48 hours, cells were harvested and lysed by three cycles of freezing and thawing in 0.1 molar potassium phosphate (KPO_4), pH 7.8, containing 1mM dithiothreitol. The cell extracts were assayed for luciferase activity as described in Example 4. Western blot analysis revealed that the hybrid DNA construct, Luc23 cDNA-genomic DNA, synthesizes native luciferase.

It will be apparent to those skilled in the art that there are numerous variations in the actual processes and materials that can be used to effect the purposes of the subject invention. Therefore, the present examples should be considered as instructional only and are not to be interpreted to limit the invention.

We claim:

CLAIMS

1. A method for producing hybrid molecules useful in biological assays exhibiting luciferase activity and a second functionality comprising forming an association of three sequences of DNA by isolating a first sequence of DNA encoding luciferase activity, and associating with said first sequence of DNA a second sequence of DNA encoding said second functionality; and

associating said first and second sequences of DNA with a third sequence of DNA encoding functions allowing for selecting, replicating, and expressing said hybrid molecules in a suitable host cell; and

inserting said association of three sequences of DNA into said suitable host cell; and

selecting and replicating said host cells; and

isolating from said host cells said hybrid molecules.

2. A method as described in Claim 1 wherein isolating said first sequence of DNA comprises constructing either a cDNA or a genomic library from bioluminescent organisms exhibiting single subunit luciferases;

and selecting from said cDNA or said genomic library said first sequence of DNA encoding said luciferase activity.

3. A method as described in Claim 2 wherein said first sequence of DNA encodes luciferase activity within a sequence of about 1.8 kilobases bounded by Eco RI endonuclease restriction sites.

4. A method as described in Claim 1 wherein said second sequence of DNA is associated with said first sequence of DNA comprising being adjacent to or within said first sequence of DNA providing for functional transcribing of said hybrid protein molecule encoded by said first and said second sequences of DNA.

5. A method as described in Claim 4 wherein said second sequence of DNA encoding said second functionality encodes a protein substance having antigenic and/or enzymatic and/or binding activity.

6. A method as described in Claim 1 wherein said third sequence of DNA encoding functions allowing for selecting, replicating, and expressing said hybrid molecules in suitable host cells is functional in either procaryotic and/or eucaryotic host cells.

7. A method for producing molecules useful in biological assays exhibiting luciferase activity comprising forming an association of two sequences of DNA by isolating a first sequence of DNA encoding luciferase activity and associating said first sequence of DNA with a second sequence of DNA encoding functions allowing for selecting, replicating, and expressing said molecules with luciferase activity in a suitable host cell; and

inserting said association of two sequences of DNA into said suitable host cell; and

selecting and replicating said host cell; and

isolating said molecules with said luciferase activity.

8. A method as described in Claim 7 wherein isolating said first sequence of DNA comprises constructing either a cDNA or a genomic library from bioluminescent organisms exhibiting single subunit luciferases;

selecting from said DNA or said genomic library said first sequence of DNA encoding said luciferase activity.

9. A method as described in Claim 8 wherein said first sequence of DNA encodes luciferase activity within a sequence of about 1.8 kilobases of DNA bounded by Eco RI restriction endonucleases sites.

10. A method as described in Claim 7 wherein said second sequence of DNA encoding functions allowing for selecting, replicating, and expressing said luciferase molecules in a suitable host cell is functional in either procaryotic and/or eucaryotic host cells.

11. A hybrid plasmid comprising three functionally distinct sequences of DNA wherein said first sequence encodes a biological molecule exhibiting luciferase activity, said second sequence encodes a biological molecule lacking luciferase, and said third sequence encodes functions allowing for selecting, replicating, and expressing said plasmid in a suitable host cell.

12. Procaryotic and/or eucaryotic cells including hybrid plasmids as described in Claim 11.

13. Hybrid proteins generated from hybrid plasmid as described according to Claim 11.

14. A hybrid plasmid comprising two functionally distinct sequences of DNA wherein said first sequence encodes a biological molecule exhibiting luciferase activity, said second sequence encodes functions allowing for selecting, replicating, and expressing said plasmid in a suitable host cell.

15. A hybrid plasmid as described in Claim 14 wherein said plasmid is pKW106 and has ATCC No. 53342.

16. Procaryotic cells including the hybrid plasmid as described in Claim 15.

17. Proteins with luciferase activity generated from said hybrid plasmid described in Claim 15.

18. Immunoassay for determining the concentration of antigenic substances in fluids comprising affixing antibodies directed against antigenic determinants of said antigenic substances to a solid surface, forming a mixture by contacting said solid surface in an aqueous solution containing fluid with an unknown amount of said antigenic substances and a hybrid molecule exhibiting at least one of said antigenic determinants of said antigenic substances and luciferase activity, incubating said mixture under conditions of controlled time, temperature, pH, and concentration of said antibody bound to said solid surfaces to allow bonding of said antigenic substances and said hybrid molecules to said antibody, separating said solid surfaces containing bound antigenic substances and hybrid molecules, and determining the intensity of bioluminescence emitted from said hybrid molecules bound to said solid surfaces, and comparing that intensity with the intensity from a mixture and incubation containing a known concentration of said antigenic substances under the same conditions used in the assay of said antigenic substances.

19. Immunoassay as described in Claim 18 wherein said intensity of bioluminescence is measured comprising adding to said solid surfaces luciferin, ATP, molecular oxygen, and measuring the intensity of bioluminescence.

20. Immunoassay as described in Claim 19 wherein said antigenic substances are selected from the group consisting of polypeptides, proteins, or polymers or derivatives thereof.

21. A method for producing hybrid molecules useful in biological assays exhibiting luciferase activity and a second functionality comprising forming an association of three sequences of DNA wherein said first sequence is cDNA encoding luciferase activity, said second sequence is genomic DNA encoding translational start, and said third DNA sequence is vector DNA encoding functions allowing for selecting, replicating, and expressing said hybrid molecules in a suitable host cell; and
inserting said association of three sequences of DNA into said suitable host cell; and
selecting and replicating said host cells; and
isolating from said host cells said molecules with luciferase activity.

22. A method as described in Claim 21 wherein isolating said first sequence of DNA comprises constructing a cDNA library isolating said second sequence from a genomic DNA library from bioluminescent organisms exhibiting single subunits luciferases and selecting from said cDNA library said first region of DNA encoding said luciferase activity.

23. A method as described in Claim 21 wherein said genomic DNA is a 110 - 120 base 5' Eco RI sequence derived from a Photinus pyralis genomic DNA library Kilobase cDNA sequence described in Claim 2.

24. A method as described in Claim 21 wherein said suitable host cells are procaryotes and/or eucaryotes.

25. A method as described in Claim 21 wherein said suitable host cells are plant cells.

26. Hybrid plasmids comprising three sequences of DNA wherein said first sequence is cDNA encoding luciferase activity and polyadenylation sites, said second sequence is genomic DNA encoding translational start sites, and said third sequence is vector DNA encoding functions allowing for selecting, replicating, and expressing said plasmid in a suitable host cell.

27. A hybrid plasmid as described in Claim 26, wherein said plasmid is pJD201 with ATCC No. 53341.

28. Cytochemical assays for cell surface or cytoplasmic associated molecules comprising incubating in solution said cell surface or cytoplasmic associated molecules with hybrid molecules exhibiting luciferase activity and binding activity to said cell surface or cytoplasmic associated molecules under conditions of suitable pH, ionic strength, and temperature to effect binding of said hybrid molecules, washing said cell surface or cytoplasmic associated molecules to remove unbound hybrid molecules, and revealing bound hybrid molecules by incubating said cell surface or cytoplasmic associated molecules with said bound hybrid molecules in a solution with adenosine triphosphate, luciferase, and oxygen.

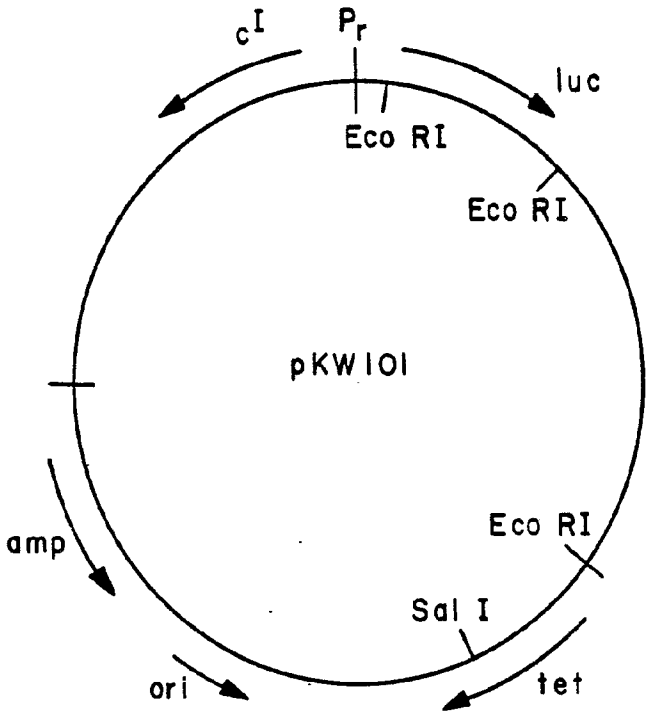
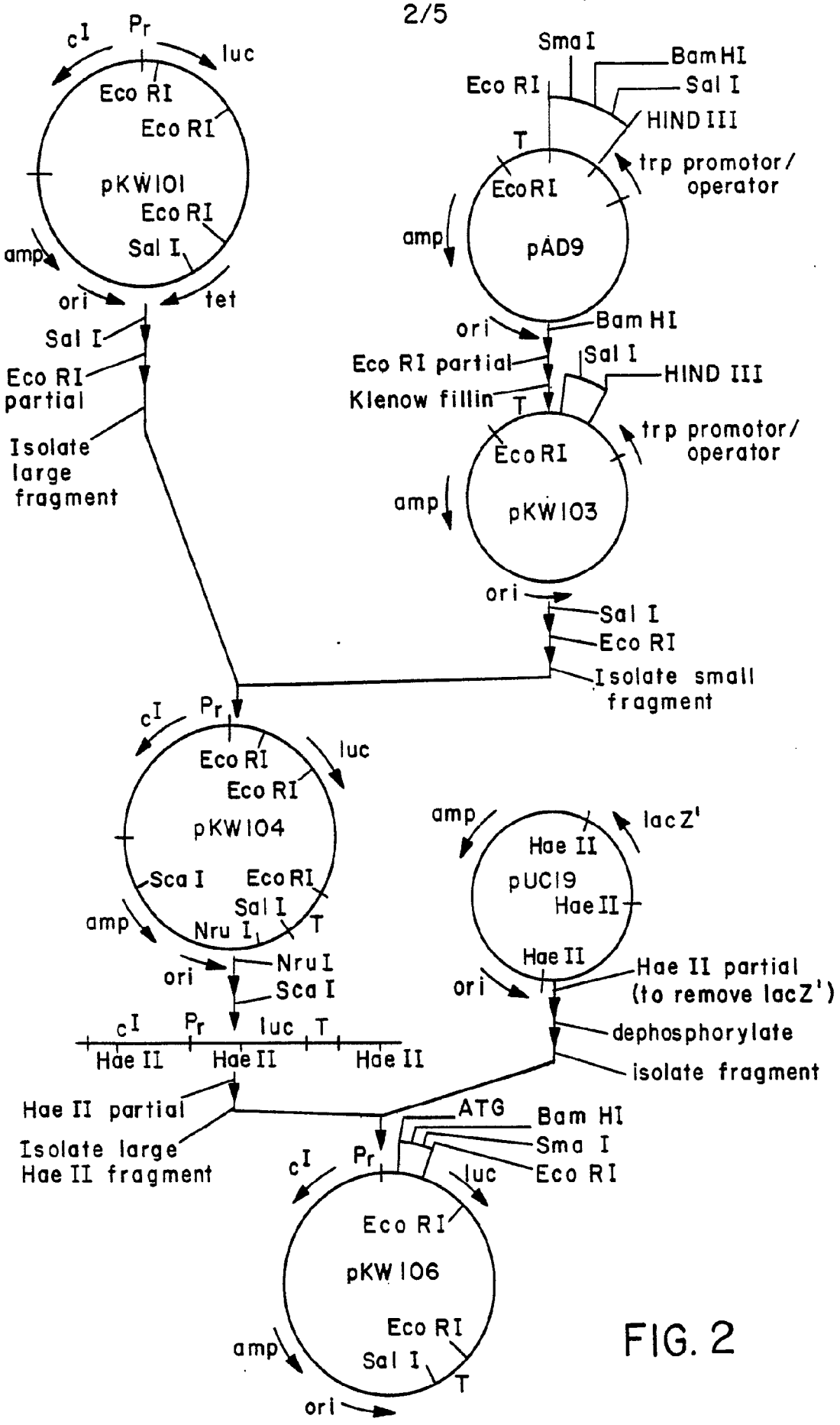


FIG. 1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Bam HI
Eco RI
Sal I
HIND III
T
trp promotor/ operator
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amp
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Eco RI partial
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cI
Pr
luc
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Eco RI
pKW104
Sca I
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Sal I
amp
Nru I
T
Nru I
ori
Sca I
cI
Pr
luc
T
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Hae II
Hae II
Hae II partial
Isolate large Hae II fragment
amp
lacZ'
Hae II
pUC19
Hae II
Hae II
ori
Hae II partial (to remove lacZ')
dephosphorylate
isolate fragment
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ori

FIG. 2

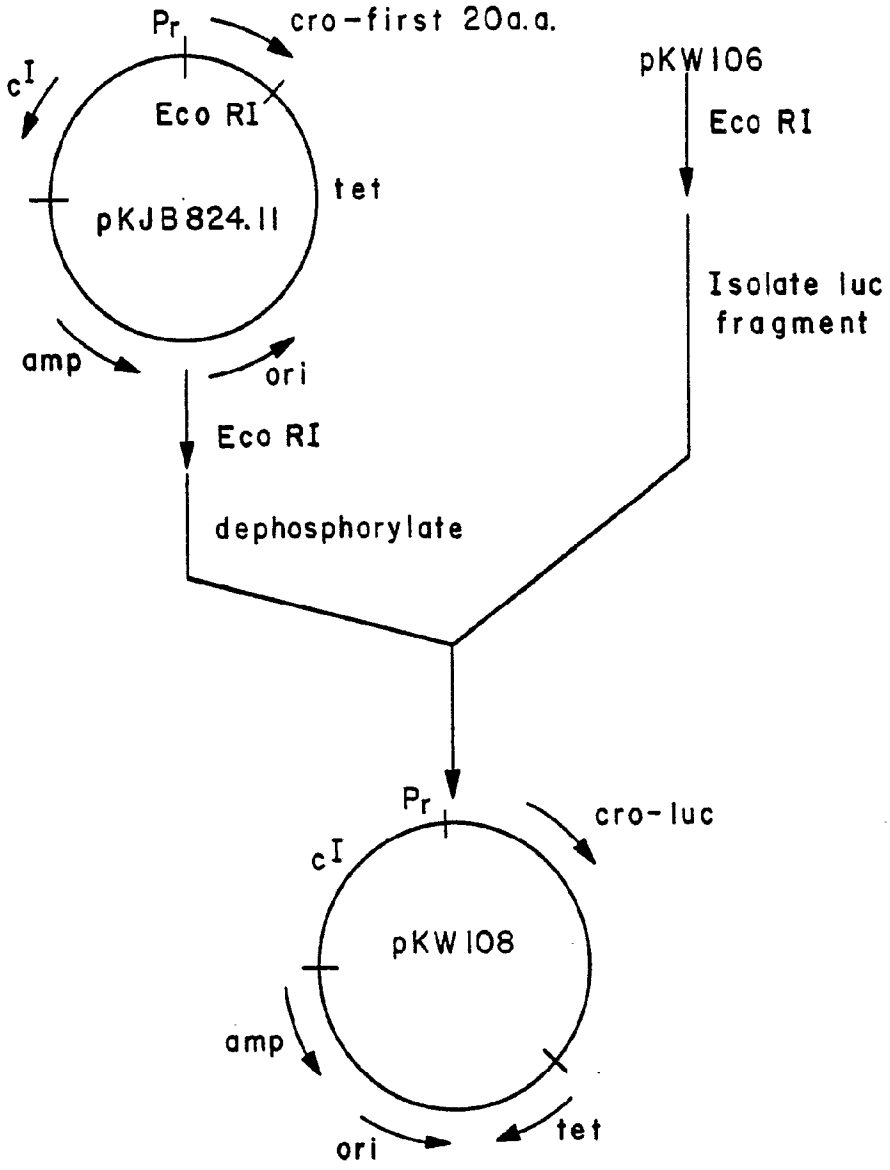
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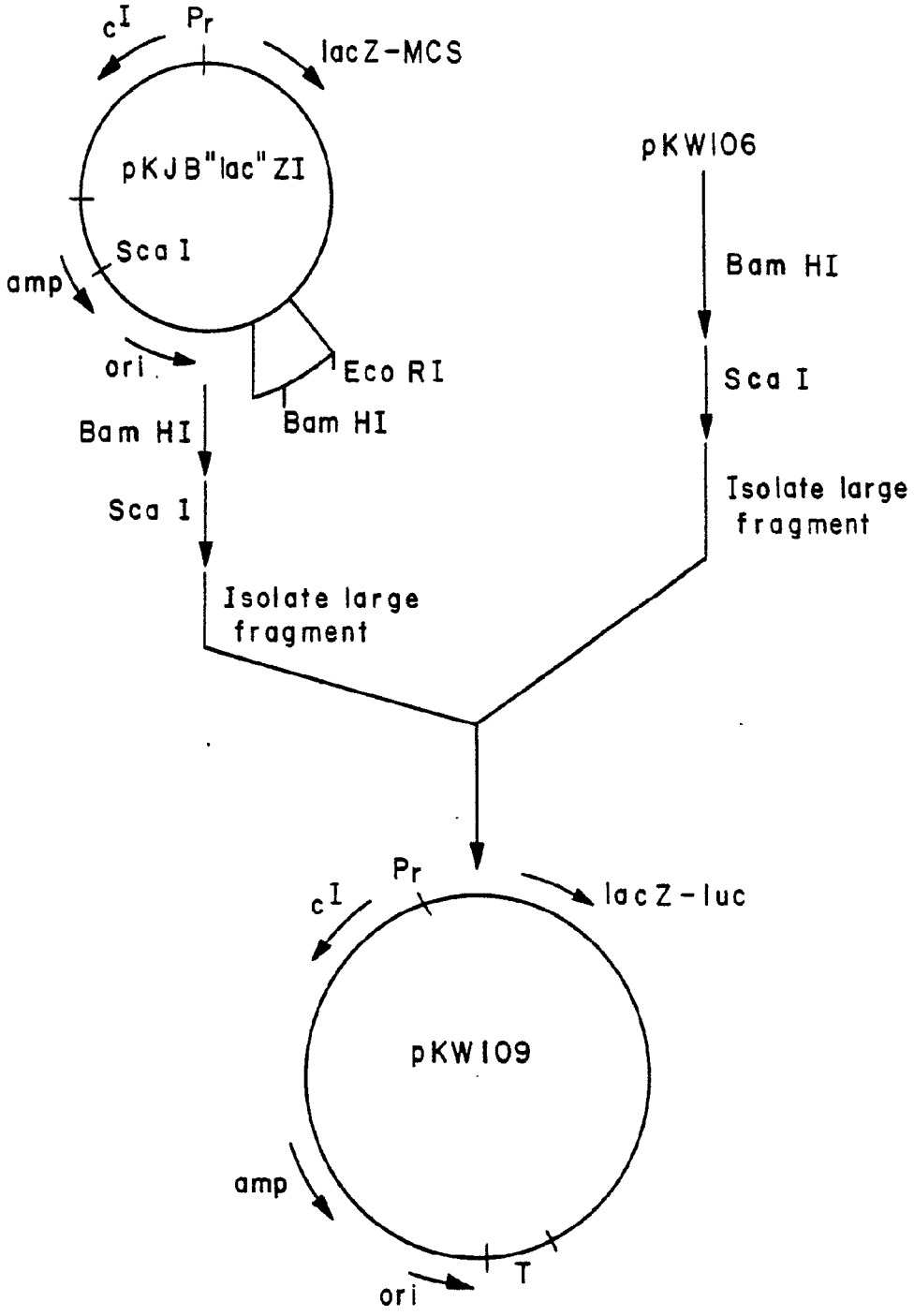
FIG. 3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ori
T

FIG. 4

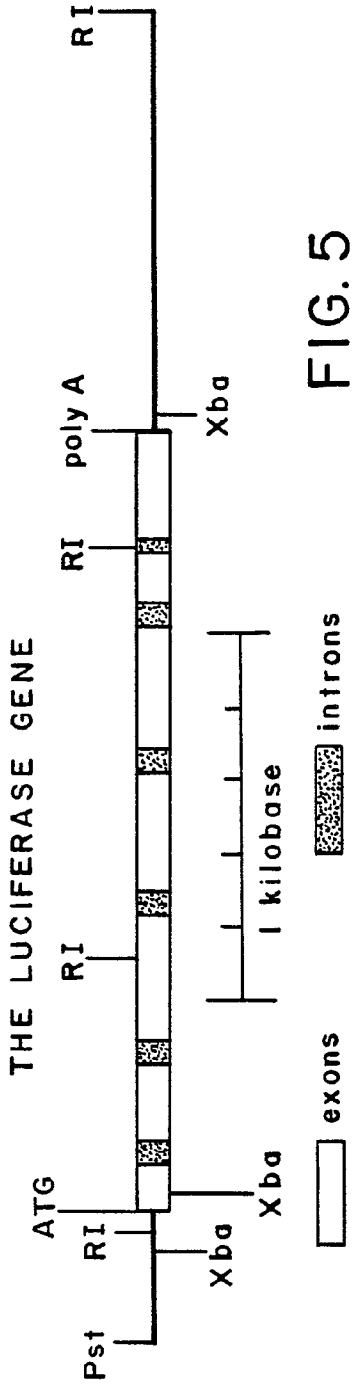

FIG. 5

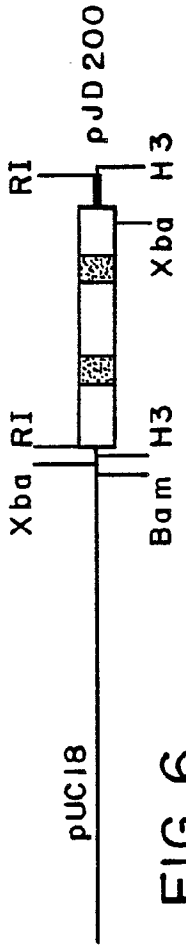

FIG. 6

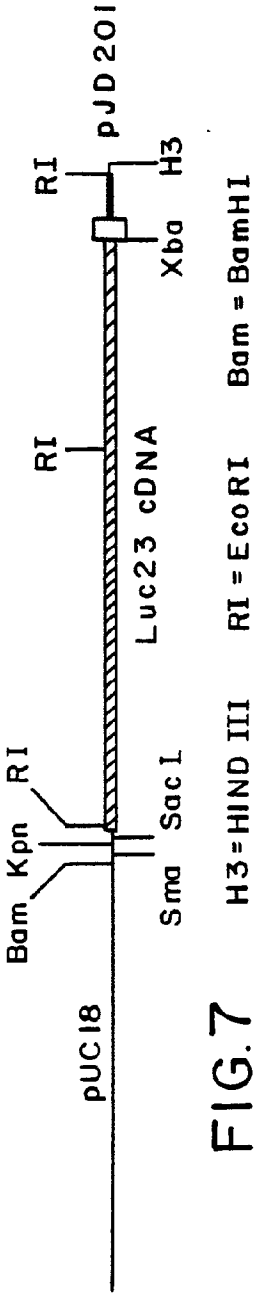

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02589

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: C12P 21/00, C12N 15/00, C12N 1/00
US: 435/68, 172.3, 317

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68, 172.3, 189, 240, 253, 255, 317 935/11, 12, 14, 15, 27, 47, 69, 70, 72

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACT DATA BASE (CAS) 1967-1987
BIOSIS 1969-1987 See attachment for keywords.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y,P	U.S., A 4,581,335 (BALDWIN) 8 April 1986. See Abstract and columns 1 and 3.	1-6 & 11-13
Y	Journal of Biological Chemistry, Volume 260, No. 10, issued May, 1985 (Baltimore, Maryland, U.S.A.) (D.H. COHN et al) "Nucleotide Sequence of the lux A Gene of Vibrio harveyi and the Complete Amino Acid Sequence of the α-Subunit of Bacterial Luciferase", See pages 6139-6146, see specifically 6139 and 6141.	1-6 & 11-13
X	Journal of Bacteriology, Volume 164, No. 1, issued October, 1985 (Washington, D.C., U.S.A.) (P.V. DUNLAP et al) "Control of Vibrio fischeri Luminescence Gene Expression in Escherichia coli by Cyclic AMP and Cyclic AMP Receptor Protein", See pages 45-50, see specifically pages 45, 46 and 48.	1,2,4-6 & 11-13

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
06 February 1987	02 MAR 1987
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Jayme A. Huleatt

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Cell, Volume 32, issued March 1983 (Cambridge, Massachusetts, U.S.A.) (J. ENGEBRECHT et al) "Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from Vibrio fischeri", See pages 773-781, see specifically pages 773, and 776-778.	1,2,4-6 & 11-13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Group I - Claims 1-6 and 11-13.

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

II. FIELDS SEARCHED KEYWORDS CONTINUED

Luciferase, Beta galactocidase, clone, cloning, plasmid, vector, fuse, fusion, fusing, hybrid, immunoassay, assay, oxidoreductase, lac Z, lux

PCT/ISA/206 INVITATION

Group I- Claims 1-6 and 11-13, drawn to a method for producing hybrid molecules coded for by three DNA sequences where the first DNA sequence codes for luciferase activity, the second DNA sequence codes for a second DNA function and the third DNA sequence codes for functions which allow host replication and hybrid plasmids and transformed cells containing these three DNA sequences, classified in 435/68, 172.3, 240, 253, 255 and 317.

Group II- Claims 7-10 and 14-17, drawn to a method of producing molecules coded for by two DNA sequences where the first DNA sequence codes for a protein with luciferase activity and the second sequence codes for functions which allow host replication, hybrid plasmids and transformed cells containing these two DNA sequences and proteins with luciferase activity, classified in 435/68, 172.3, 240, 253, 255, 317 and 189.

Group III- Claims 18-20, drawn to an immunoassay; classified in 435/7.

Group IV- Claims 21-27, drawn to a method for producing hybrid molecules coded for by three DNA sequences where the first DNA sequence codes for luciferase activity, the second DNA sequence is a translational start and the third DNA sequence codes for functions which allow host replication and hybrid plasmids containing these three DNA sequences, classified in 435/68, 172.3, 240 and 317.

Group V- Claim 28 is drawn to a cytochemical assay, classified in 435/7.

Groups I,II and IV are related as methods of making different hybrid molecules, utilizing hybrid plasmids containing the appropriate DNA sequences. Although all three of the methods utilize recombinant techniques, the methods are distinct because each method utilizes distinct plasmids to produce the corresponding hybrid molecules.

Groups III and V are separate and distinct because Group III requires the use of an antibody in determining antigenic substances in fluids whereas Group V is a different assay, only requiring a binding activity for determining cell surface or cytoplasmic associated molecules.

Groups I, II and IV are separate and distinct from Groups III and V because the assays of Groups III and V can be practiced with molecules other than those produced by Groups I, II and IV, such as other enzymes conjugated to antibodies or other molecules with binding activity.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
Y	Proceedings National Academy of Sciences U.S.A, Volume 80, issued March 1983 (Washington, D.C., U.S.A.) (R.A. YOUNG et al) "Efficient Isolation of Genes by Using Antibody Probes", See pages 1194-1198, see specifically pages 1194-1196 and 1198.	1-6 & 11-13
Y	Chemical Abstracts, Volume 102, No. 3, issued 1985 January 21 (Columbus, Ohio, U.S.A.), (D. HAENGGI) "Agent for Antigen/Antibody Detection", see page 361, column 1, the abstract no. 20786p, Ger. Offen. DE 3,314,812.	1-6 & 11-13
X	Journal of Bacteriology, Volume 158, No. 3, issued June, 1984 (Washington, D.C., U.S.A) (R. BELAS et al) "Transposon Mutagenesis of Marine Vibrio spp.", See pages 890-896, see specifically pages 890 and 893-894.	1,2,4-6 & 11-13